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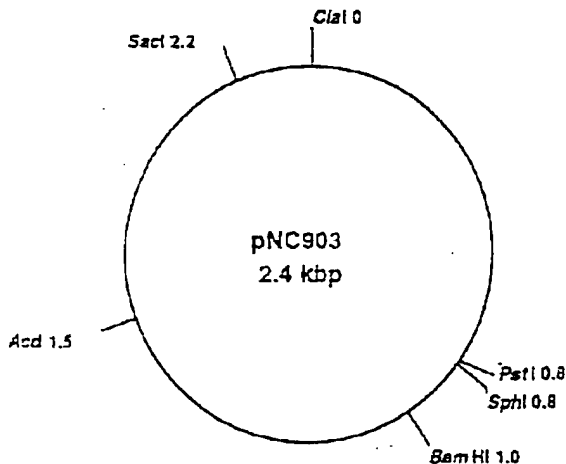
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(57) 【要約】

【構成】 分子量が約2.4kbの環状プラスミドであり、制限酵素 *EcoRI*、*XbaI*、*KpnI*、*HindIII*、*BglII*、又は *SpeI* によって切断されず、制限酵素 *Clal*、*PstI*、*SphI*、*BamHI*、*AccI*、及び *SacI* によって切断される部位数がそれぞれ1であり、且つ前記の制限酵素 *Clal*、*PstI*、*SphI*、*BamHI*、*AccI*、及び *SacI* によって切断される部位が図1の制限酵素開裂地図で示されるプラスミドpNC903。

【効果】 環状プラスミドpNC903は、それに含まれる種々の制限酵素による開裂部位を利用して、外来のDNA断片を導入修飾し、多くの有用なプラスミドベクターの開発に利用でき、特に、ノカルディオフォルム細菌を宿主とする宿主-ベクター系におけるプラスミドベクターとして有用である。



【特許請求の範囲】

【請求項1】 分子量が約2.4kbの環状プラスミドであり、制限酵素 *EcoRI*、*XbaI*、*KpnI*、*HindIII*、*BglII*、又は *SpeI* によって切断されず、制限酵素 *Clal*、*PstI*、*SphI*、*BamHI*、*AccI*、及び *SacI* によって切断される部位数がそれぞれ1であり、且つ前記の制限酵素 *Clal*、*PstI*、*SphI*、*BamHI*、*AccI*、及び *SacI* によって切断される部位が下記の制限酵素開裂地図化1で示されるプラスミドpNC903。

(74) [Attorney(s) Representing All Applicants]

[Patent Attorney]

(57) [Abstract]

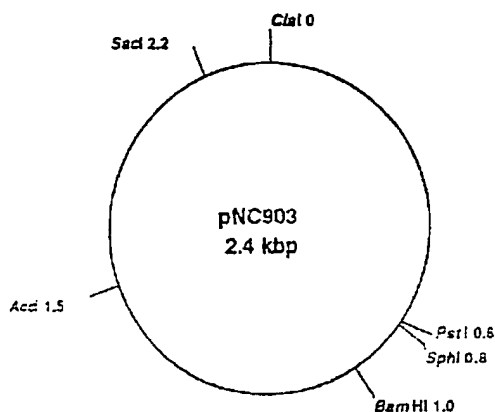
[Constitution] Molecular weight being ring shape plasmid of approximately 2.4 kb, to be, restriction enzyme *EcoRI*, *XbaI*, *KpnI*, *HindIII*, *BglII*, Or quantity of site which is not cut off by *SpeI*, is cut off by restriction enzyme *Clal*, *PstI*, *SphI*, *BamHI*, *AccI*, and *SacI* is 1 respectively, plasmid pNC903 where site which is cut off and aforementioned restriction enzyme *Clal*, by *PstI*, the *SphI*, *BamHI*, *AccI*, and *SacI* is shown with the restriction enzyme cleavage map of Figure 1.

[Effect(s)] Ring shape plasmid pNC903 be able to introduce and be able to decorate imported DNA fragment, be able to utilize in development of many useful plasmid vector making use of cleavage site due to various restriction enzyme which is included in that, especially, it is useful as plasmid vector in host-vector system which designates no cult D. off a jpl1 Δ bacteria as host.

[Claim(s)]

[Claim 1] Molecular weight being ring shape plasmid of approximately 2.4 kb, to be, restriction enzyme *EcoRI*, *XbaI*, *KpnI*, *HindIII*, *BglII*, Or quantity of site which is not cut off by *SpeI*, is cut off by restriction enzyme *Clal*, *PstI*, *SphI*, *BamHI*, *AccI*, and *SacI* is 1 respectively, plasmid pNC903 where site which is cut off and aforementioned restriction enzyme *Clal*, by *PstI*, the *SphI*, *BamHI*, *AccI*, and *SacI* is shown with the below-mentioned restriction enzyme cleavage map Chemical Formula 1.

【化 1】



【請求項 2】 該環状プラスミドが、*Rhodococcus rhodochrous* P-II-123-1 (工業技術院生命工学工業技術研究所 寄託番号 FERM P-14193) 由来のプラスミドであることを特徴とする請求項 1 に記載のプラスミド pNC903。

【発明の詳細な説明】

【0001】

【産業上の利用分野】 本発明は、新規なプラスミドに関し、具体的には、エポキシド生産菌である *Rhodococcus* 属に属する微生物に由来する新規な環状プラスミドに関する。本発明の環状プラスミドは、それに含まれる制限酵素によって切断される部位を利用して、両端に当該制限酵素によって切断される DNA 配列を有する DNA 断片を連結してなる人工の環状プラスミドの調製に用いることができる。

【0002】

【従来の技術】 短鎖長オレフィン炭素源として、土壤中より分離したノカルディア属、ロドコッカス属といったノカルディオフォルム細菌はオレフィンを酸化して、対応するエポキシド類を生産することが知られている。例えば、プロピレン炭素源として土壤中より分離したノカルディア・コラリーナは、光学活性なエポキシドや、合成樹脂、医薬、農薬などの有機化学製品の製造原料中間体として広範囲に利用できるトリフルオロプロペンオキシド (TFPO) の生産などに利用されている。これらの土壤中より分離したノカルディオフォルム細菌を用い、更なる菌株改良のため、エポキシド生産能を有するノカルディオフォルム細菌の宿主-ベクター系の開発が以前から期待されていた。これらの微生物を宿主とするに適したベクター開発は遅

[Chemical Formula 1]

[Claim 2] Said ring shape plasmid, plasmid pNC903 which is stated in Claim 1 which designates that it is a plasmid of *Rhodococcus rhodochrous* P-II-123-1 (Agency of Industrial Science and Technology National Institute of Bioscience and Human-Technology deposit number FERM P-14193) derivation as feature.

[Description of the Invention]

[0001]

[Field of Industrial Application] This invention regards novel ring shape plasmid which derives in microorganism which belongs to *Rhodococcus* being attached which concretely, is an epoxide producing microbe in regard to the novel plasmid. Making use of site which is cut off by restriction enzyme which is included in that, connecting DNA fragment which possesses DNA sequence which is cut off to both ends by this said restriction enzyme you can use for manufacturing the artificial ring shape plasmid which becomes ring shape plasmid of this invention.

[0002]

[Prior Art] With short chain long olefin as carbon source, as for no cult. D. off. *jp114* bacteria such as *Nocardia* and *Rhodococcus* sp. which are separated from the soil oxidation doing olefin, it is known that it produces the epoxide which corresponds. *Nocardia* * コラリーナ which is separated from in soil with the for example propylene as carbon source is utilized production etc of trifluoropropene oxide (TFPO) which can be utilized in broad range in optically active epoxide and synthetic resin, pharmaceutical and as the process raw material intermediate of pesticide or other organic chemical

れているが、本発明者は、既にエポキシド生産株のノカルディア・コラリーナより、ノカルディオフォルム細菌の宿主ベクター系に適用可能なプラスミドpNC500を見だし、特許出願した（特開 平 5-244953 号公報を参照）。また、ロドコッカス属の一部を宿主とする、宿主ベクター系の開発例として、ジャーナル オブ バクテリオロジー (J. Bacteriol.) 170, 638 (1988)、アプライドアンド エンバイロメンタル マイクロバイオロジー (Appl. Environ. Microbiol.) 56, 2818 (1990)、プラスミド (Plasmid) 23, 242 (1990) 等の数例が報告されている。

[0003] しかしながら、前記するプラスミドpNC500を除き、従来より報告されているノカルディオフォルム細菌由来のプラスミドの多くは、ロドコッカス属の一部のみを宿主にするにすぎないものであった。そのため、より広い範囲のノカルディオフォルム細菌を宿主として適用でき、宿主のエポキシド生産菌から、微生物を育種、改良するための新しいベクターの開発が強く要望されている。

[0004]

[発明が解決しようとする課題] 本発明は上記の課題を解決するもので、本発明の目的は、ノカルディオフォルム細菌を宿主として、ノカルディオフォルム細菌中において複写が可能である新規な環状プラスミドを提供することにある。特に、ノカルディオフォルム細菌に類別されるロドコッカス属に属する微生物に由来する新規な環状プラスミドDNAを提供することにある。

[0005]

[課題を解決するための手段] 本発明者らは、ノカルディオフォルム細菌を宿主とでき、DNA組換えに使用可能な新規なプラスミドDNAを開発すべく鋭意研究を行ったところ、当該細菌に類別され、エポキシド生産菌であるロドコッカス属に属する微生物から、宿主ベクター系におけるベクターとして利用可能な新規な環状プラスミドを見出し、本発明を完成した。

[0006] 本発明のプラスミドは、分子量が約 2.4 kb の環

product. Making use of no cull D. off お jp11 Δ bacteria which is separated from soil of these, for strain improvement on that of development of host-vector system of no cull D. off お jp11 Δ bacteria which possesses epoxide production ability was expected from time before. Designates these microorganism as host vector development which issued is late, but this inventor, from Nocardia * Corally Δ of the epoxide producing strain, you discovered applicable plasmid pNC500 already in host-vector system of no cull D. off お jp11 Δ bacteria, patent application did (Japan Unexamined Patent Publication Hei 5-244953 disclosure reference). In addition, portion of Rhodococcus sp. is designated as host, the journal of bacteriology (Journal of Bacteriology (0021-9193, JOBAAY)) 170, 638 (1988) and Applied and Environmental Microbiology (Applied and Environmental Microbiology (0099-2240, AEMIDF)) 56, 2818 (1990), plasmid (Plasmid) 23, 242 (1990) or other several examples is reported as development example of host-vector system

[0003] But, many of plasmid of no cull D. off お jp11 Δ bacteria derivation which is reported from until recently excluding plasmid pNC500 which description above is done, only designate only portion of the Rhodococcus sp. as host were something. Because of that, be able to apply no cull D. off お jp11 Δ bacteria, of wider range, as host from epoxide producing microbe of host, in order breeding, to improve microorganism development of new vector is strongly demanded.

[0004]

[Problems to be Solved by the Invention] As for this invention being something which solves above-mentioned problem, objective of this invention is to offer novel ring shape plasmid where copy is possible with no cull D. off お jp11 Δ bacteria as host, in the no cull D. off お jp11 Δ bacteria. Especially, it is to offer novel ring shape plasmid DNA which derives in microorganism which belongs to Rhodococcus sp. which classifies in no cull D. off お jp11 Δ bacteria.

[0005]

[Means to Solve the Problems] As for these inventors, no cull D. off お jp11 Δ bacteria in order that it can make host, develops useable novel plasmid DNA in DNA rearrangement when the diligent research was done, was classified in this said bacteria, from microorganism which belongs to Rhodococcus sp. which is a epoxide producing microbe, practical novel ring shape plasmid was discovered as the vector in host-vector system, this invention was completed.

[0006] As for plasmid of this invention, molecular weight

状プラスミドであり、制限酵素 EcoRI、XbaI、KpnI、HindIII、BglII、又は SpeI によって切断されず、制限酵素 ClaI、PstI、SphI、BamHI、AccI、及び SacI によって切断される部位数がそれぞれ1であり、且つ前記の制限酵素 ClaI、PstI、SphI、BamHI、AccI、及び SacI によって切断される部位が下記の制限酵素開裂地図化1で示されるプラスミドであり、該プラスミドにプラスミドpNC903の呼称を本発明者は付与する。

【化1】

【0007】本発明のプラスミドpNC903を保有する微生物として、具体的にロドコッカス属に属する微生物であり、プロピレン酸化性菌として土壌から分離した、エポキシド生産能を有する菌株 *Rhodococcus rhodochrous* P-II-123-1 (工業技術院生命工学工業技術研究所 寄託番号 FERM P-14193) が挙げられる。なお、この微生物 *Rhodococcus rhodochrous* P-II-123-1 は、上記の寄託番号 FERM P-14193 で通産省工業技術院生命工学工業技術研究所に寄託されており、また、該菌株の菌学的性質は表1に示すとおりである。

【0008】

【表1】

菌学的性質		
グラム染色性		+
孢子		-
運動性		-
形状	桿菌	
コロニーの形状	ピンクオレンジ、不透明、円形、規則性、凸状、直径 0.5mm以下 (3日間培養)	
各温度での生育	37℃	+
	45℃	(+)
カタラーゼ		+
オキシダーゼ		-
OFテスト		-

【0009】細胞の化学分析

ミコール酸を含む。細胞壁のジアミノ酸はメソ-DAPである。脂肪酸成分は、16:0, 16:1, 18:1, 10-Me・18:0及び微量の14:0, 15:0, 17:0から成っている。

ght being ring shape plasmid of approximately 2.4 kb, to be, restriction enzyme EcoRI, XbaI, KpnI, HindIII, BglII, Or it is cut off by SpeI, restriction enzyme ClaI, PstI, Quantity of site which is cut off by SphI, BamHI, the AccI, and SacI is 1 respectively, is plasmid where site which is cut off and aforementioned restriction enzyme ClaI, by PstI, SphI, BamHI, AccI, and the SacI is shown with below-mentioned restriction enzyme cleavage map Chemical Formula 1, naming plasmid pNC903 it grants this inventor to said plasmid.

[Chemical Formula 1]

[0007] It was a microorganism which concretely belongs to *Rhodococcus* sp. as microorganism which possesses plasmid pNC903 of this invention, you can list strain *Rhodococcus rhodochrous* P-II-123-1 (Agency of Industrial Science and Technology National Institute of Bioscience and Human-Technology deposit number FERM P-14193) which it separated from soil as propylene-feeding microbe, possesses epoxide production ability. Furthermore, this microorganism *Rhodococcus rhodochrous* P-II-123-1 with above-mentioned deposit number FERM P-14193 the deposit is done in Ministry of International Trade and Industry Agency of Industrial Science and Technology National Institute of Bioscience and Human-Technology, in addition, microbiological characteristic of said strain is as shown in Table 1.

[0008]

[Table 1]

[0009] Chemical analysis of cell

Mycolic acid is included. diamino acid of cell wall is meso-DAP. aliphatic acid component has consisted of 14:0, 15:0, 17:0 of 16:0, 16:1, 18:1, 10-Me・18:0 and

【0010】生化学テスト

分解

アデニン	+
チロシン	+
尿素	-

単一炭素源での生育

イノシトール ¹	-
マルトース ¹	(+)
マンニトール ¹	+
ラムノース ¹	-
ソルビトール ¹	+
m-ヒドロキシ安息香酸 ²	+
アジピン酸塩 ²	+
安息香酸塩 ²	+
クエン酸塩 ²	+
乳酸塩 ²	+
グルタミン酸塩 ²	-
L-チロシン ²	+
グリセロール ¹	(+)
トレハロース ¹	(+)
p-ヒドロキシ安息香酸 ²	+
D-マンノース ¹	-
アセトアミド ²	+
D-ガラクトース ¹	-

酵素活性

α -グルコシダーゼ	+
システインアリルアミダーゼ	-
バリンアリルアミダーゼ	-

生育テスト (存在下)

5% NaCl	+
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trace amount.

[0010] Biochemistry test

Disassembly

Adenine	+
Tyrosine	+
Urea	-

Growth with single carbon source

Inositol 1	-
Maltose 1	(+)
Mannitol 1	+
Rhamnose 1	-
Sorbitol 1	+
M - hydroxybenzoic acid 2	+
Adipate 2	+
Benzoate 2	+
Citrate 2	+
Lactate 2	+
Glutamate 2	-
L- tyrosine 2	+
Glycerol 1	(+)
Trehalose 1	(+)
P- hydroxybenzoic acid 2	+
D- mannose 1	-
Acetamide 2	+
D- galactose 1	-

Enzyme activity

- glucosidase	+
Cysteine allyl amidase	-
Valine allyl amidase	-

Growth test (Under of existence)

5% NaCl	+
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アジドナトリウム (0.02% w/v) -

【0011】注：表中の肩表記する記号は、培地に対する添加濃度を示し、また、符号(+)は下記の水準を意味する。

【0012】1. 1 % (w/v)

2. 0.1 % (w/v)

(+) weak positive

【0013】本発明のプラスミドpNC903は、具体的には上記微生物 *Rhodococcus rhodochrous* P-II-123-1 から、次に示す方法により分離することができる。

【0014】ノカルディオフォルム細菌は、通常の培地中で培養するとき、リゾチームなどの溶菌酵素で溶菌されにくいので、予めグリシン入り培地で当該微生物を培養し、ペニシリンGで処理することにより、溶菌酵素で溶菌され易い菌を得る。例えば、下記の組成のNBG培地にグリシンを1% (w/v) 程度添加した培地が用いられる。

(NBG培地)

Nutrient Broth No.2 (Oxoid) 2.5 % (w/v)

グルコース 1.0 % (w/v)

当該微生物を30℃で対数増殖後期まで培養し、更にペニシリンG所定量を培地に添加し数時間培養を継続した後、溶菌酵素（リゾチーム、アクロモバクターペプチターゼ等）で溶菌する。得られる溶菌液より、環状プラスミドDNAは、公知の方法で分離することができる（*Biochimica. et Biophysica. Acta.*, 383, 457-463 (1975) を参照）。更に、分離された環状プラスミドDNAの集合から、アガロースゲル上で電気泳動することにより、分子量の異なる環状プラスミドDNAを分離し、目的の分子量を有する環状プラスミドDNAを単離することができる。なお、当該微生物 *Rhodococcus rhodochrous* P-II-123-1 は、環状プラスミドDNAとして、分子量が約2.4 kbの該プラスミドpNC903 一種のみを有しており、容易に単離することができる。

【0015】分子量が約2.4 kbの環状プラスミドとして単離される当該プラスミドpNC903の有する種々の制限酵素に対する感受性を調べると、その感受性は表2に示すものである。

Azido sodium(0.02% w/v) -

[0011] Note: Shoulder notation of in the table symbol which is done shows the addition concentration for culture medium, in addition, symbol (+) means below-mentioned level.

[0012] 1. 1 % (w/v)

2. 0.1 % (w/v)

(+) Weak positive

[0013] It can separate plasmid pNC903 of this invention, due to method which from the above-mentioned microorganism *Rhodococcus rhodochrous* P-II-123-1, is shown concretely next.

[0014] Because no cult. of *N. f.* bacteria is difficult to be done, when culturing in conventional culture medium, lysis with lysozyme or other bacteriolytic enzyme, this said microorganism is cultured beforehand with glycine entering culture medium, microbe which the lysis is easy to be done is obtained with bacteriolytic enzyme by treating with the penicillin G. 1 % (w/v) extent is added culture medium which can use glycine for NBG culture medium of the for example below-mentioned composition.

(NBG culture medium)

Nutrient Broth No.2 (Oxoid) 2.5 % (w/v)

Glucose 1.0 % (w/v)

With 30 °C it cultures this said microorganism to logarithmic proliferation post phase, furthermore add the penicillin G predetermined amount to culture medium and after continuing several hours culture, lysis is done with bacteriolytic enzyme (Such as lysozyme and *Achromobacter* peptidase). From lysate which is acquired, it can separate cyclic plasmid DNA, with known method (*Biochimica. et Biophysica. Acta.*, 383, 457-463 (1975) reference). Furthermore, cyclic plasmid DNA where molecular weight differs from gathering of the cyclic plasmid DNA which is separated, by electrophoresis doing on agarose gel, can be separated, cyclic plasmid DNA which possesses molecular weight of object can be isolated. Furthermore, as for this said microorganism *Rhodococcus rhodochrous* P-II-123-1, molecular weight has had only the said plasmid pNC903 one kind of approximately 2.4 kb as cyclic plasmid DNA, can isolate easily.

[0015] When molecular weight sensitivity for various restriction enzyme which this said plasmid pNC903 which is isolated as ring shape plasmid of

approximately 2.4 kb has is inspected,
thesensitivity is something which is shown in Table 2.

【0016】

【表2】

プラスミドpNC903の制限酵素に対する感受性

制限酵素	切断個所の数
EcoRI	0
XbaI	0
KpnI	0
HindIII	0
BglII	0
SpeI	0
ClaI	1
PstI	1
SphI	1
BamHI	1
AccI	1
SacI	1

【0017】なお、上記制限酵素は、それぞれ次の菌種から得られる制限酵素である。

制限酵素	菌種の名称
EcoRI	Escherichia coli RY13
XbaI	Xanthomonas badrii (ATCC 11672)
KpnI	Klebsiella pneumoniae OK8
HindIII	Haemophilus influenzae Rd
BglII	Bacillus globigii
SpeI	Sphaerotilus natans (ATCC 13923)
ClaI	Caryophanon latum L
PstI	Providencia stuartii 1641 pPst101
SphI	Streptomyces phaeochromogenes

[0016]

[Table 2]

It confronts restriction enzyme of plasmid pNC903 sensitivity

Number of restriction enzyme	cutting site
EcoRI	0
XbaI	0
KpnI	0
HindIII	0
BglII	0
SpeI	0
ClaI	1
PstI	1
SphI	1
BamHI	1
AccI	1
SacI	1

[0017] Furthermore, above-mentioned restriction enzyme is restriction enzyme which is acquired from respective following microbe type.

Title of restriction enzyme	microbe type
EcoRI	Escherichia coli RY13
XbaI	Xanthomonas badrii (ATCC 11672)
KpnI	Klebsiella pneumoniae OK8
HindIII	Haemophilus influenzae Rd
BglII	Bacillus globigii
SpeI	Sphaerotilus natans (ATCC 13923)
ClaI	Caryophanon latum L
PstI	Providencia stuartii 1641 pPst101
SphI	Streptomyces phaeochromogenes

BamHI *Bacillus subtilis* MT-2 (pBamHI RM22)
 AccI *Acinetobacter calcoaceticus*
 SacI *Streptomyces achromogenes* (ATCC 12767)

【0018】各制限酵素による切断個所の数は、過剰量の制限酵素存在下にプラスミドpNC903を完全消化し、得られる消化物（切断されたDNA）を0.7%アガロースゲル電気泳動にかけ、分離可能なDNA断片の種類数から決定される。大腸菌のラムダファージのDNA（λ DNA）を制限酵素 HindIII で消化し得られる、既知分子量のDNA断片（J. Mol. Biol., 98, 551-564 (1975)を参照）を同一のアガロースゲル上での電気泳動にかけ、その泳動距離で描かれる標準曲線に基づき、切断されたDNA断片の分子量は算出した。上記の制限酵素 ClaI、PstI、SphI、BamHI、AccI、又は SacI で完全消化し、得られる消化物（切断されたDNA）は、それぞれ約 2.4 kb の分子量を有する単一のDNA断片であった。更に、該プラスミドpNC903を、上記の制限酵素 ClaI、PstI、SphI、BamHI、AccI、SacI の2種以上を組み合わせ用いた処理によって得られる、複数のDNA断片の分子量をそれぞれアガロースゲル電気泳動で測定することにより、図1に示す制限酵素開裂地図を作成した。なお、互いに近接して存在する PstI と SphI の開裂位置は、アガロースゲル上での電気泳動による分子量の測定では容易に区別できないが、PstI と BamHI により切断される約 0.2 kb DNA断片中に SphI の切断部位が存在することを確認することで PstI と SphI の開裂位置の関係を特定した。また、本発明のプラスミドpNC903は、その由来するノカルディオフォルム細菌（*Rhodococcus rhodochrous* P-11-123-1）中において複製が行え、ノカルディオフォルム細菌のDNA複製開始点（レプリコン）を内在している。

【0019】本発明のプラスミドpNC903は、図1に示す制限酵素による開裂部位を有しており、該開裂部位を利用して、該プラスミドを修飾して種々の有用なプラスミドベクターを開発することができる。更には、該プラスミド或はその修飾により得られる誘導体に、目的とする遺伝子のDNA断片、例えば、モノオキシゲナーゼ等の酵素或は蛋白質をコードする遺伝子のDNA断片を上記の制限酵素による開裂部位を利用して組み込み、得られる環状プラスミドを宿主微生物に導入して、宿主を形質転換させることが可能である。当該プラスミドpNC903を宿主ベクター系として適用できる宿主微生物としては、該プラスミドpNC903が由来する *Rhodococcus rhodochrous* P-11-123-1 と類似するDNA鎖合成酵素（DNAポリメラーゼ）など

BamHI *Bacillus subtilis* MT-2 (pBamHI RM22)
 AccI *Acinetobacter calcoaceticus*
 SacI *Streptomyces achromogenes* (ATCC 12767)

[0018] Plasmid pNC903 complete digestion it does quantity of cutting site due to each restriction enzyme, under restriction enzyme existing of excess quantity, it applied the digest (It was cut off DNA) which is acquired on 0.7% agarose gel electrophoresis, is decided from the quantity of types of separable DNA fragment. digestion it does DNA (DNA) of lambda phages of *E. coli* with restriction enzyme HindIII and is acquired, you applied DNA fragment (Journal of Molecular Biology (0022-2836, JMOBAC), 98, 551-564 (1975) reference) of known molecular weight on the electrophoresis on same agarose gel, you calculated molecular weight of DNA fragment which is cut off on basis of standard curve which is drawn with phoresis distance. Above-mentioned restriction enzyme ClaI, complete digestion it did with PstI, the SphI, BamHI, AccI or SacI, digest (It was cut off DNA) which is acquired was single DNA fragment which possesses molecular weight of the respective approximately 2.4 kb. Furthermore, restriction enzyme cleavage map which is shown in Figure 1 by being acquired by treatment which is used said plasmid pNC903, above-mentioned restriction enzyme ClaI, combining 2 kinds or more of PstI, SphI, BamHI, AccI and the SacI measures molecular weight of DNA fragment of multiple respectively with agarose gel electrophoresis, was drawn up. Furthermore, proximity doing mutually, cleavage position of the PstI and SphI which exist cannot distinguish with the measurement of molecular weight with electrophoresis on agarose gel easily, but it was related cleavage position of PstI and SphI by fact that you verify that cleavage site of SphI exists in approximately 0.2 kb DNA fragment which is cut off by PstI and BamHI specific. In addition, as for plasmid pNC903 of this invention, duplication does, the DNA replication starting point (replicon) of no cull D. off *jp11* Δ bacteria is indwelling that in in the no cull D. off *jp11* Δ bacteria (*Rhodococcus rhodochrous* P-II-123-1) which derives.

[0019] Plasmid pNC903 of this invention cleavage site due to restriction enzyme which is shown in the Figure 1 has had, making use of said cleavage site, decorating said plasmid, the various useful plasmid vector can develop. Furthermore, in derivative which is acquired said plasmid or by the decoration, DNA fragment of gene which is made object, to install the DNA fragment of gene which for example monooxygenase or other enzyme or protein code is done making use of cleavage site due to above-mentioned restriction enzyme, introducing cyclic plasmid which is acquired into host microorganism,

ラスミドDNAの複製を司る酵素群を有するノカルディオフォルム細菌が好適に用いられる。なお、該プラスミドpNC903を宿主ベクター系として適用するに際しては、該プラスミドを汎用される選択マーカーの遺伝子、例えば、放線菌由来のチオストレプトン耐性遺伝子、大腸菌由来のクロラムフェニコール耐性遺伝子、アンピシリン耐性遺伝子、カナマイシン耐性遺伝子などの薬剤耐性をもたらす遺伝子DNAにより修飾して、耐性遺伝子を内在したプラスミドベクターとして用いると好ましい。即ち、該プラスミドpNC903を修飾して得られる耐性遺伝子を内在したプラスミドベクターに目的の遺伝子のDNA断片を組み込み得られる環状プラスミドを作製し、この環状プラスミドを目的とする宿主微生物に導入して、前記の薬剤耐性を指標とし選別することで、容易に該環状プラスミドにより形質転換された微生物を選別することができる。

【0020】なお、上記の該プラスミドpNC903の耐性遺伝子による修飾、或はポリペプチドや蛋白質をコードする遺伝子などのDNA断片の組み込みは、公知の遺伝子DNA組み換え技術、例えば、参照文献 Scientific American 233(1) 24-33 (1975)、Molecular Cloning: a Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor (1982)などに記載されている手法を用いて行うことができ、目的とする環状プラスミドはその分子量を量り分取することができる。また、本発明のプラスミドpNC903、或は該プラスミドpNC903から上記のDNA組み換え技術により調製される環状プラスミドを宿主微生物に導入するには、例えば、宿主微生物であるノカルディオフォルム細菌の細胞をプロトプラスト或はスフェロプラストにして、環状プラスミドをポリエチレングリコール(PEG)と共存させることで環状プラスミドを移入する方法(J. Bacteriol. 170, 638-645 (1988)などを参照)、または細胞を対数増殖期中期まで培養し、高電圧の電気パルスを与えて環状プラスミドを移入するエレクトロポレーション法(Appl. Environ. Microbio. 56, 2818-2825 (1990)などを参照)等の公知の技術を用いることができる。

【0021】更には、本発明のプラスミドpNC903をプラスミドベクターとして用い、目的とする酵素或は蛋白質をコードする

neoplastic transformation the host it is possible to do. It can use for ideal no cull D. off jp11 Δ bacteria which possesses the enzyme group which administers duplication of plasmid DNA such as the DNA chain synthase (DNA polymerase) which resembles Rhodococcus rhodochrous P-II-123-1 where said plasmid pNC903 derives as host-vector system the this said plasmid pNC903 as host microorganism which it can apply. Furthermore, when said plasmid pNC903 when it applies, as host-vector system decorating gene of selectable marker which said plasmid is widely used, the thio pick-up β ton resistance gene of for example Actinomycetes derivation, chloramphenicol resistance gene of E. coli derivation, with genetic DNA which brings ampicillin resistance gene and kanamycin resistance gene or other drug resistance, it uses resistance gene as plasmid vector which is indwelling, it is desirable. Namely, decorating said plasmid pNC903, resistance gene which is acquired it produces the cyclic plasmid which installs DNA fragment of gene of object in the plasmid vector which is indwelling, is acquired, introducing into host microorganism which designates this cyclic plasmid as object, it designates the aforementioned drug resistance as indicator and by fact that it sorts easily it can sort microorganism which neoplastic transformation is done due to said cyclic plasmid.

[0020] Furthermore, as for installation of gene or other DNA fragment which decoration or the polypeptide and protein due to resistance gene of above-mentioned said plasmid pNC903 the code is done, it is possible to do genetic DNA rearranging technology of the public knowledge, making use of technique which is stated in for example cited reference Scientific American 233(1) 24-33 (1975) and the Molecular Cloning: a Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor (1982) etc cyclic plasmid which is made objective measures molecular weight and the fraction collection is possible. In addition, plasmid pNC903 of this invention, Or cyclic plasmid which is manufactured from said plasmid pNC903 by the above-mentioned DNA rearranging technology is introduced into host microorganism, cyclic plasmid method which imports cyclic plasmid by fact that it coexists with polyethylene glycol (PEG) (Journal of Bacteriology (0021-9193, JOBAAY) 170, 638-645 (1988) etc reference), or cell is cultured to logarithmic growth phase midperiod the cell of no cull D. off jp11 Δ bacteria which is a for example host microorganism to protoplast or spheroplast, electric pulse of high voltage is given and electroporation method (Appl. Environ. Microbio. 56, 2818-2825 (1990) etc reference) or other known technology which imports cyclic plasmid can be applied.

[0021] Furthermore, With plasmid pNC903 of this invention as plasmid vector to use, It is possible to

遺伝子を組み込み得られる環状プラスミドを導入した宿主微生物の形質転換は、それ自体を公知の方法で培養して、目的とする酵素或は蛋白質を産出させることができ、又目的とする酵素とその基質との反応による有用な代謝物の製造を行うことができる。即ち、目的とする酵素或は蛋白質を細胞外に分泌生産させたり、或は細胞内に生産蓄積させたりでき、更には生産させた酵素により、例えば、基質のオレフィンを酵素により酸化して、対応する光学活性なエポキシド類を生産するなどの酵素反応を行ったり、難分解物質を酵素反応により分解して代謝物に変換したりすることができる。

[0022]

[実施例 1]

Rhodococcus rhodochrous P-II-123-1 からのプラスミド pNC903 の単離

Rhodococcus rhodochrous P-II-123-1 のスラントより 3 白金耳量を採取し、それを液体培地 [Nutrient Broth No. 2 (Oxid) 2.5 % (w/v), グルコース 1 % (w/v), グリシン 1 % (w/v)] 500ml に接種し、30°C で 21 時間振盪培養した。この時点で、前記培養液中における濃度が 0.5 units/ml となる量のペニシリン G を添加し、さらに 30°C で 3 時間培養を継続した。次いで、培養した当該菌の菌体を該培養液から集菌し、TE 緩衝液 [0.025M トリス (ヒドロキシメチル) アミノメタン (トリス)、0.025M EDTA : pH8.0] で洗浄した。該菌体を、溶菌液 [0.3M ショ糖、0.025M トリス、0.025M EDTA、2 mg/ml リゾチーム、2 mg/ml アクロモペプチダーゼ、50 µg/ml RNase : pH8.0] 20ml 中に懸濁し、30°C で 2 時間反応させた。得られる反応液に、2% (w/v) ラウリル硫酸ナトリウムと 0.3M 水酸化ナトリウムからなる溶液 10ml を添加し、良く混合してから 55°C の湯浴中に 1 時間置いた。この液に、フェノール・クロロホルム (1 容 : 1 容) 混合液 4ml を加え、1 分間良く混ぜ、液全体を白濁させた。

[0023] この白濁した液を、4°C で 30 分間 17000 × g の遠心分離にかけ、得られる上層の溶液を分取した。再度、それと等量のフェノール・クロロホルム (1 容 : 1 容) 混合液を加え良く混ぜた後、4°C で 30 分間 17000 × g の遠心分離にかけ、分離した上層の溶液を分取した。

produce useful metabolite with enzyme which is made the objective or to install gene which protein code is done as for the neoplastic transformation strain of host microorganism which introduces cyclic plasmid which is acquired, culturing that itself with known method, it it makes objective of the enzyme or it to be possible fact that it produces protein, with the reaction of enzyme and substrate which in addition it makes the objective. Namely, enzyme or, protein secretory production doing or product accumulation be able to do, furthermore with enzyme which is produced in extracellular, the olefin of for example substrate oxidation doing with enzyme, or other enzymatic reaction which produces optically active epoxide which correspond to do, difficultly digested matter quality which is made the objective disassembling with enzymatic reaction, it can convert to metabolite in the intracellular.

[0022]

[Working Example 1]

Isolation of plasmid pNC903 from Rhodococcus rhodochrous P-II-123-1

3 platinum loop quantity it recovered from slant of Rhodococcus rhodochrous P-II-123-1, that the inoculation did in liquid culture medium (Nutrient Broth No. 2 (Oxid) 2.5 % (w/v), glucose 1 % (w/v), glycine 1 % (w/v)) 500 ml, 21 hour shaking culture did with 30 °C. With this time point, penicillin G of quantity where concentration in the aforementioned culture medium becomes 0.5 units/ml was added, furthermore 3 hours culture continued with 30 °C. Next, microbe collection it did cell mass of this said microbe which was cultured from the said culture fluid, washed with TE buffer (0.025M tris (hydroxymethyl) aminomethane (tris) and 0.025 MED TA: pH 8.0). Suspension it did said cell mass, in lysate (0.3M sucrose, 0.025M tris, 0.025 MED TA, 2 mg/ml lysozyme, 2 mg/ml achromopeptidase (EC 3.4.21.50) and 50 g/ml RNase: pH 8.0) 20 ml, 2 hours reacted with 30 °C. in reaction mixture which is acquired, to add solution 10 ml which consists of 2 % (w/v) sodium lauryl sulfate and 0.3M sodium hydroxide, after mixing well, 1 hour you put in warm bath of 55 °C. In this liquid, 1 minute it mixed well including phenol * chloroform (1 permitting/inserting: 1 permitting/inserting) mixed solution 4 ml, the clouding did total liquid.

[0023] Liquid which this clouding is done, was applied on centrifugal separation of the 30-minute 17000 X g with 4 °C, solution of top layer which is acquired fraction collection was done. For second time, after mixing well that and including phenol * chloroform (1 permitting/inserting: 1 permitting/inserting) mixed solution of equivalent, you applied on centrifugal

【0024】この分取した上層の溶液に、それと等量のジエチルエーテルを加え、穏やかに攪拌した後しばらく放置した。分離する上層のジエチルエーテルを捨て、再度下層の溶液に、それと等量のジエチルエーテルを加え抽出した。前記のエーテル抽出後、得られる下層の液に、0.1倍容の3M 酢酸ナトリウム水溶液及び2倍容のエタノールを加え、析出した沈殿物を遠心分離で回収した。回収した沈殿物を5mlのTE緩衝液に溶解し、更に CsCl 7.5g と、1.5mg/ml 臭化エチジウム-TE緩衝液2ml とを加え混合し、溶液を得た。この溶液を42時間 120,000 × gの密度勾配遠心分離にかけた。

【0025】遠心分離したプラスミド画分を、紫外線照射により検出し、分取した。分取したプラスミド画分を、n-ブタノールで処理し臭化エチジウムを除いた。その後、TE緩衝液に対して透析し、エタノール沈殿により精製プラスミド画分を得た。該精製プラスミド画分を 0.5 mlのTE緩衝液に溶解し、0.7%アガロースゲル電気泳動(100 V、1時間)に供し、分子量 約 2.4 kb のプラスミドバンドの存在を確認した。該分子量 約 2.4kb のプラスミドバンドを含むゲル部分を分取した。このゲル部分を少量のTE緩衝液とともに透析膜に入れ、電気泳動させ、更に逆方向に短時間泳動させた後、透析膜内に残る溶液を回収し、この回収した溶液に、0.1倍容の3M 酢酸ナトリウム水溶液及び2倍容のエタノールを加え、析出した沈殿物を遠心分離で回収して、真空乾燥を行い、目的のプラスミドpNC903を得ることができる。なお、上記の精製プラスミド画分をアガロースゲル電気泳動に供し、分子量の異なるプラスミドを分離する際、前記分子量 約 2.4kb のプラスミドバンド以外のプラスミドバンドは見出されず、当該菌の有する環状プラスミドは、プラスミドpNC903のみであることが判る。

【0026】また、プラスミドpNC903を、各種制限酵素によって単一消化、二重消化、或いは三重消化し、得られるDNA断片をアガロースゲル電気泳動にかけ、各DNA断片の移動度から分子量を求めたところ、プラスミドpNC903の分子量は約 2.4 kb であった。この場合、制限酵素の反応条件は、供給者に

separation of 3 0-minute 17000 X g with the 4 °C, fraction collection you did solution of top layer which is separated.

[0024] In solution of top layer which this fraction collection is done, after agitating calmly, that and including diethyl ether of equivalent, it left for awhile. You threw away diethyl ether of top layer which it separates, for thesecond time in solution of bottom layer, you extracted that andincluding diethyl ether of equivalent. After aforementioned ether extraction, in liquid of bottom layerwhich is acquired, precipitate which was precipitated including the 0.1 time permitting/inserting 3M sodium acetate aqueous solution and 2-foldpermitting/inserting ethanol, it recovered with centrifugal separation. it melted precipitate which recovers in TE buffer of the5 ml, it mixed furthermore including with CsCl 7.5g and 1.5 mg/ml ethidium bromide-TE buffer 2 ml ,acquired solution. This solution was applied on density gradient centrifugal separation of 4 2 hours 120,000 X g.

[0025] Centrifugal separation it detected plasmid fraction which is done, with ultraviolet light illumination, the fraction collection did. plasmid fraction which fraction collection is done, was treated with n- butanol and theethidium bromide was excluded. dialysis it did after that, vis-a-vis TE buffer, itacquired refining plasmid fraction with ethanol precipitation. It melted said refining plasmid fraction in TE buffer of 0.5 ml, offeredto 0.7% agarose gel electrophoresis (100 V and 1 hour), verified existence of plasmid band of molecular weight approximately 2.4 kb . gel portion which includes plasmid band of said molecular weight approximately 2.4 kb the fraction collection was done. With TE buffer of trace inserting this gel portion in dialysis membrane, the electrophoresis doing, furthermore with centrifugal separation solution which in reverse directionremains after short time phoresis and inside dialysis membrane it recovers, this in thesolution which recovers, precipitate which it precipitated including the 0.1 time permitting/inserting 3M sodium acetate aqueous solution and 2-foldpermitting/inserting ethanol, recovering, vacuum drying it does, plasmid pNC903of objective can acquire. Furthermore , above-mentioned refining plasmid fraction is offeredto agarose gel electrophoresis, when separating plasmid where molecular weight differs, theplasmid band other than plasmid band of aforementioned molecular weight approximately 2.4 kb is not discovered, as for cyclic plasmid which this said microbe has, onlythe plasmid pNC903 it understands that is.

[0026] In addition, plasmid pNC903, when single digestion, double digestion or triple digestionit did with various restriction enzyme, it applied DNA fragmentwhich is acquired on agarose gel electrophoresis, sought molecular weight from mobility

よって定められた条件に従った。なお、分子量は、 λ DNAを制限酵素 HindIII で分解して得られる断片の標準移動度のパターンを基にして決定した。更に、これらの結果から、該プラスミド pNC903は図1の制限酵素地図を示すことが分かった。また、アガロースゲル上での電気泳動による分子量の測定では容易に区別できない、互いに近接して存在する PstIと SphIの開裂位置は、PstIと BamHIにより切断される約 0.2 kb DNA断片中に SphIの切断部位が存在することを確認することで PstIと SphIの開裂位置の関係を特定した。

【0027】

【発明の効果】本発明の環状プラスミド pNC903は、種々の制限酵素による開裂部位各一つを有しており、この特定される開裂部位を利用して、外来の DNA断片を導入修飾し、多くの有用なプラスミドベクターを開発することができる。更に、当該プラスミドは、ノカルディオフォルム細菌を宿主として、ノカルディオフォルム細菌中において複写が可能である、宿主ベクター系におけるプラスミドベクターとして有用である。また、該プラスミドを用いて、外来の DNA断片を導入修飾して得られる環状プラスミドベクターとし、更に得られる環状プラスミドベクターに種々の酵素或は蛋白質をコードする遺伝子を組み込み得られる環状プラスミドを導入した宿主微生物の形質転換株を利用し、該形質転換株を培養して目的の酵素或は蛋白質を産出させることができ、又目的とする酵素とその基質との反応による有用な代謝物や酵素反応産物の製造を行うことができる。

【図面の簡単な説明】

【図1】 制限酵素 ClaI、PstI、SphI、BamHI、AccI、及び SacIによる切断部位の相対位置を示すプラスミド pNC903の制限酵素開裂地図。

of each DNA fragment, molecular weight of plasmid pNC903 was approximately 2.4 kb. In this case, reaction condition of restriction enzyme, you followed condition which is decided by supplier. Furthermore, disassembling DNA with restriction enzyme HindIII, the pattern of standard mobility of fragment which is acquired it decided the molecular weight, on basis of. Furthermore, from these results, as for said plasmid pNC903 it understood that the restriction enzyme map of Figure 1 is shown. In addition, you cannot distinguish with measurement of molecular weight with electrophoresis on agarose gel easily, proximity doing mutually, it was located PstI and SphI which exist cleavage, by fact that you verify that cleavage site of SphI exists in approximately 0.2 kb DNA fragment which is cut off by PstI and BamHI relationship of cleavage position of PstI and SphI specific.

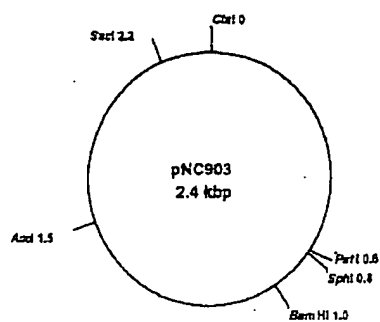
[0027]

[Effects of the Invention] Cyclic plasmid pNC903 of this invention has had cleavage site each one due to various restriction enzyme, making use of cleavage site which this specific is done, can introduce and decorate imported DNA fragment, can develop many useful plasmid vector. Furthermore, as for this said plasmid, copy is possible with no culturing of bacteria as host, in no culturing of bacteria, it is useful as plasmid vector in host-vector system. In addition, said plasmid using, introducing decorating imported DNA fragment, cyclic plasmid vector which is acquired to do, Furthermore it is possible to produce useful metabolite and enzymatic reaction product with the various enzyme or to install gene which protein code is done making use of neoplastic transformation strain of host microorganism which introduces cyclic plasmid which is acquired, culturing said neoplastic transformation strain, enzyme of objective or it is possible, to produce protein, with reaction of enzyme and the substrate which in addition it makes objective in cyclic plasmid vector which is acquired.

[Brief Explanation of the Drawing(s)]

[Figure 1] Restriction enzyme cleavage map of plasmid pNC903 which shows relative position of cleavage site due to the restriction enzyme ClaI, PstI, SphI, BamHI, AccI, and the SacI.

【図 1】



[Figure 1]

4

DDDDD	UU	UU	444	55555	666	00000
DD DD	UU	UU	4444	55	66	00 00
DD DD	UU	UU	44 44	5555	66	00 000
DD DD	UU	UU	44 44	55	66666	00 0000
DD DD	UU	UU	4444444	55	66 66	0000 00
DD DD	UU	UU	44	55 55	66 66	000 00
DDDDD	UUUUUU		4444	5555	6666	00000

2222	00000	00000
22 22	00 00	00 00
22	00 000	00 000
222	00 0000	00 0000
22	0000 00	0000 00
22 22	000 00	000 00
222222	00000	00000

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Use of SYCO for catalyst optimization

SYCO is a series of related plasmids in which operons relevant for 3G production are expressed by promoters of varying strengths on a single plasmid. The operons may include, but are not limited to, *dhaB1-3,X*, *orfY,X,W*, and *dar1/gpp2*. Individual operons may also be complete or may be missing one or more of the genes that make up the operon. The promoters include, but are not limited to, the GI promoter mutants previously described. The various expression cassettes (promoter-operon) are modular and can be assembled in a wide variety of configurations. This allows direct analysis of the impact of expression level on catalyst performance, and therefore facilitates catalyst optimization.

EXAMPLE

Use of SYCO to examine effect of orf expression on catalyst performance

The orf operon from pKP32 (described previously) was PCR amplified (SEQ ID NOS: 6-7) with *HindIII* at the 5' end and *AvrII* at the 3' end, and subcloned between *HindIII* and *AvrII* in pLitmus28 (New England Biolabs) to generate pKP38. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P1.6 from pMP38/1.6 (described elsewhere) was subcloned between *EcoRI* and *HindIII* in pKP38 to generate pKP39. The *AvrII/XbaI* restriction fragment containing the *dhaB* expression cassette from pMP38/1.6 was subcloned between *AvrII* and *XbaI* in pLitmus28 (New England Biolabs) to generate pMP39. The *AvrII/XbaI* restriction fragment containing the *dhaB* expression cassette from pMP39 was subcloned into the *AvrII* site of pRJ50 to generate pSYCO11. The *AvrII* restriction fragment containing the orf expression cassette from pKP39 was subcloned into the *NheI* site of pSYCO11 to generate pSYCO12. The plasmids pSYCO11 and pSYCO12 are identical except that pSYCO11 does not contain the orf operon. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of orf expression on catalyst performance metrics such as titer (Table 1), rate, yield or B12 utilization.

Table 1

	<u>orf expression</u>	<u>3G titer (g/L)</u>
pSYCO11	no	58
pSYCO12	yes	110

EXAMPLE

Use of SYCO to examine effect of orf expression level on catalyst performance

The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter GI P1.5 from pMP38/1.5 (described elsewhere) was subcloned between *EcoRI* and *HindIII* in pKP38 to generate pKP40. The *AvrII* restriction fragment containing the orf operon driven by GI P1.5 from pKP40 was subcloned into the *NheI* site of pSYCO11 to generate pSYCO13. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P1.20 from pMP38/1.20 was subcloned between *EcoRI* and *HindIII* in pKP38 to generate pKP41.

The *AvrII* restriction fragment containing the orf expression cassette from pKP41 was subcloned into the *NheI* site of pSYCO11 to generate pSYCO14. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P3.4 from pMP38/3.4 was subcloned between *EcoRI* and *HindIII* in pKP38 to generate pKP47. The *AvrII* restriction fragment containing the orf expression cassette from pKP47 was subcloned into the *NheI* site of pSYCO11 to generate pSYCO24. The plasmids pSYCO12, pSYCO13, pSYCO14 and pSYCO24 are identical except for the specific GI promoter mutant used to express the orf operon, which is P1.6, P1.5, P1.20 and P3.4, respectively. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of orf expression level on catalyst performance metrics such as titer (Table 2), rate, yield or B12 utilization.

Table 2

	<u>relative orf expression</u>	<u>3G titer (g/L)</u>
pSYCO12	1.0	110
pSYCO13	0.25	80
pSYCO14	0.0625	95
pSYCO24	6.6	88

EXAMPLE

Use of SYCO to examine effect of *orfY* expression on catalyst performance

The *orfX-orfW* genes were PCR amplified using primers designed to incorporate a *HindIII* site at the 5' end and an *AvrII* site at the 3' end (SEQ ID NOS: 1-2) from pKP32 and the product was subcloned between *HindIII* and *AvrII* in pLitmus28 (New England Biolabs) to generate pKP37. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P1.6 from pMP38/1.6 was subcloned between *EcoRI* and *HindIII* in pKP37 to generate pKP43. The *AvrII* restriction fragment containing P1.6 expressing *orfX-orfW* from pKP43 was subcloned at *NheI* in pSYCO11 to generate pSYCO15. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P1.5 from pMP38/1. was subcloned between *EcoRI* and *HindIII* in pKP37 to generate pKP44. The *AvrII* restriction fragment containing P1.5 expressing *orfX-orfW* from pKP44 was subcloned at *NheI* in pSYCO11 to generate pSYCO16. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P1.20 from pMP38/1.20 was subcloned between *EcoRI* and *HindIII* in pKP37 to generate pKP45. The *AvrII* restriction fragment containing P1.20 expressing *orfX-orfW* from pKP45 was subcloned at *NheI* in pSYCO11 to generate pSYCO17. The *EcoRI/HindIII* restriction fragment containing the GI mutant promoter P3.4 from pMP38/3.4 was subcloned between *EcoRI* and *HindIII* in pKP37 to generate pKP48. The *AvrII* restriction fragment containing P3.4 expressing *orfX-orfW* from pKP48 was subcloned at *NheI* in pSYCO11 to generate pSYCO25. The plasmids pSYCO12 and pSYCO15 are identical except that pSYCO15 does not express *orfY*. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of *orfY* expression on catalyst performance metrics such as titer (Table 3), rate, yield or B12 utilization.

Table 3

	<u>orfY expression</u>	<u>3G titer (g/L)</u>
pSYCO12	yes	110
pSYCO15	no	122

SEQ ID NO: 1

CAT AAG CTT AAC GAG GGG ACC GTC ATG TCG

SEQ ID NO: 2

ACG CCT AGG CCA GTT CAA GCG CAA GCA TCA G

EXAMPLE

Use of SYCO to examine effect of glycerol dehydratase expression level on catalyst performance

The *AvrII/NotI* restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO12 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO18. The *AvrII/NotI* restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO13 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO19. The *AvrII/NotI* restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO14 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO20. The *HindIII* restriction fragment containing the orf operon followed by GI P1.5 from pSYCO19 was subcloned at *HindIII* in pMP38 to generate pSYCO26. The plasmids pSYCO13 and pSYCO19 are identical except for the specific GI promoter mutant used to express glycerol dehydratase, which is P1.6 and P1.5, respectively. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of glycerol dehydratase expression level on catalyst performance metrics such as titer, rate (Table 4), yield or B12 utilization.

Table 4

	relative		3G rate (g/tank/hr)		
	<u>dehydratase</u>	<u>2 mg B12</u>	<u>4 mg B12</u>	<u>8 mg B12</u>	<u>16 mg B12</u>
pSYCO13	1.0	2.9	11.4	37.1	35.1
pSYCO19	0.25	11.1	20.7	32.0	33.9

EXAMPLE

Use of SYCO to examine effect of glycerol dehydratase expression level in the absence of orfY expression on catalyst performance

The *AvrII/NotI* restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO15 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO21. The *AvrII/NotI* restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO16 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO22. The *AvrII/NotI* restriction fragment containing GI

P1.6 and 5' *dhaB1* in pSYCO17 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO23. The plasmids pSYCO15 and pSYCO21 are identical except for the specific GI promoter mutant used to express glycerol dehydratase, which is P1.6 and P1.5, respectively. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of glycerol dehydratase expression level in the absence of *orfY* expression on catalyst performance metrics such as titer (Table 5), rate, yield or B12 utilization.

Table 5

	<u>relative dehydratase</u>	<u>3G titer (g/L)</u>
pSYCO15	1.0	122
pSYCO21	0.25	105

EXAMPLE

Use of SYCO to examine effect of *orfW* expression on catalyst performance

The plasmids pSYCO106 and pSYCO109 are identical except that pSYCO109 does not express *orfW*. Therefore, these plasmids can be used to examine the effect *orfW* expression on catalyst performance metrics such as titer, rate, yield (Table 6) or B12 utilization.

Table 6

	<u><i>orfW</i> expression</u>	<u>3G weight yield (%)</u>
pSYCO106	yes	24
pSYCO109	no	19